SPECIFICATION

GENE ENCODING ADSEVERIN

TECHNICAL FIELD

This invention relates to a gene encoding adseverin, which is a Ca^{2+}-dependent actin filament-serving protein and has a function of regulating exocytosis, a recombinant vector containing this gene, a recombinant transformed by this vector, a process for producing adseverin by using the above-mentioned gene and a recombinant adseverin protein obtained by this process. The present invention also relates to an oligonucleotide hybridizable specifically with a base sequence encoding the adseverin protein, a method for regulating the formation of adseverin which comprises administering an oligonucleotide hybridizable specifically with a base sequence encoding the adseverin protein to an animal, and an antibody capable of recognizing the adseverin protein.

BACKGROUND ART

In many secretory cells in the resting state, secretion products such as neurotransmitters and hormones are stored in the form of granules or vesicles. When the cells receive adequate signals, these substances are released from the cells by exocytosis. In the process of exocytosis, the granules and vesicles migrate toward plasma membrane. Then they come into contact with the plasma membrane followed by fusion therewith, thus opening the membrane.

This exocytosis is tightly controlled by the concentration of intracellular free calcium $[Ca^{2+}]_i$ (Knight et al., Ann. N.Y. Acad. Sci. 493:504-523, 1987). Namely, it

is considered that in resting cells where $[Ca^{2+}]_i$ is low, exocytosis is blocked at several steps depending on $[Ca^{2+}]_i$ (Burgoyne, Biochem. Biophys. Acta 779:201-216, 1984). A number of secretory cells including chromaffin cells which are adrenal medulla secretory cells have a microfilament network composed of actin filaments under the plasma membrane which is supposed to serve as a barrier against the migration of granules and vesicles toward the plasma membrane (Cheek et al., FEBS Lett. 207:110-114, 1986; Lelkes et al., FBES Lett. 208:357-363, 1986). Prior to the release of the secretion products by exocytosis, this network is disassembled due to the increase in $[Ca^{2+}]_i$ by Ca^{2+}-dependent mechanisms (Vitale et al., J. Cell Biol. 113:1057-1067, 1991).

Actin is a globular protein with a molecular weight of 42 kD which is commonly distributed in eukaryocytes. It is a cytoskeleton protein closely relating to the contraction of muscle cells, etc. Actin monomers are polymerized to form filaments. Under the physiological ionic strength, actin undergoes polymerization *in vitro* at a ratio of about 100% so as to give filaments. In actual cells, however, various actin-regulating proteins contribute to the reversible conversion of filaments (gel) and monomers (sol) and changes occur depending on extracellular stimuli.

In bovine chromaffin cells, gelsolin, which seemingly relates directly to this process, was identified (Yin et al., Nature 281:583-586, 1979). Gelsolin shows a Ca^{2+}-dependent actin filament severing activity *in vitro* and exerts barbed end capping and nucleating activities on actin filaments.

Recently, adseverin (a protein of 74 kDa), which is similar to gelsolin in activity but different from it, was isolated from bovine adrenal medulla by Prof. Nonomura et al., Department of Pharmacology, Faculty of Medicine, University of Tokyo (Maekawa et al., J. Biol. Chem. 265:10940-10942).

Gelsolin is relatively widely distributed in various tissues and blood plasma (Stossel et al., Annu. Rev. Cell Biol. 1:353-402, 1985), while the distribution of adseverin is restricted mainly to the tissues with secretory functions (Sakurai et al., Neuroscience 38:743-756, 1990). This difference in tissue distribution of these proteins suggests that adseverin more closely relates to the secretory process (i.e., control of the release of neurotransmitters, endocrine substances or physiologically active substances) than gelsolin does. Accordingly, it is highly interesting to reveal the structure and function of adseverin to thereby clarify the role and regulatory mechanisms of actin filaments in exocytosis.

In former days, it was generally regarded that this process was regulated by fused proteins, etc. [Nishizaki, "Kaiko Hoshutsu Gesho ni okeru Saiboshitsu Tanpakushitsu no Yakuwari (Roles of Cytoplasmic Proteins in Exocytosis)", Saibo Kogaku (Cell Technology), 13:353-360, 1994]. However, Nonomura et al. newly point out in their hypothesis that this process finally depends on an interaction between actin and myosin. This hypothesis further provides an epoch-making idea that the regulation by the actin-severing protein takes place in non-muscular cells on the actin side, differing from the regulation on the myosin side by myosin light chain kinase

[Mochida, "Miosin Keisa Kinaze Shinkei Dentatsu Busshitsu Hoshutsu to sono Chosetsu ni okeru Miosin Keisa Kinaze no Yakuwari (Role of Myosin Light Chain Kinase in Release of Myosin Light Chain Kinase Neutrotransmitter and Regulation thereof)", Saibo Kogaku (Cell Technology), 13:381-388, 1994].

It is thought that actin is liberated from broken cells and induces or enhances platelet agglutination in the blood so as to trigger thrombus development (Scarborough et al., Biochem. Biophys. Res. Commun. 100:1314-1319, 1981). On the other hand, adseverin has a gelsolin-like activity (i.e., an actin filament-severing activity) *in vivo* as described above. These facts indicate that adseverin might be applicable to drugs relating to thrombus (for example, thrombosis inhibitors).

It is furthermore expected that the release of, for example, a physiologically active substance might be regulated at the gene level by administering the antisense DNA sequence constructed on the basis of the base sequence encoding adseverin. Since adseverin might closely relate to the multiplication of vascular smooth muscles, it is considered that the administration of the antisense DNA would regulate the function of adseverin to thereby inhibit the multiplication of the smooth muscles. Accordingly, it is expected that the administration of the antisense DNA of adseverin might be usable in the inhibition of angiostenosis in blood vessel transplantation in bypass operation, etc. or in the inhibition of restenosis after percutaneous transluminal coronary angioplasty (PTCA).

To use the actin-regulating protein adseverin in the medicinal purposes as described above, it is necessary to produce adseverin in a large amount and in a uniform state. However, it is difficult to obtain uniform adseverin in a large amount by the conventional method wherein adseverin is isolated from an animal tissue per se or the culture supernatant of adseverin-producing cells. It is therefore required to clarify the base sequence of the gene encoding adseverin so as to produce adseverin in a large amount by using gene recombination techniques.

An object of the present invention is to identify the base sequence of the gene encoding adseverin. Another object of the present invention is to produce adseverin in a large amount by using gene recombination techniques with the use of a recombinant vector containing the above-mentioned sequence and to construct a screening system, etc. by using the same, thus developing novel drugs. Another object of the present invention is to produce the antisense DNA on the basis of the base sequence of the gene encoding adseverin and use it as a drug for inhibiting the formation of adseverin. Another object of the present invention is to provide an antibody capable of recognizing the adseverin protein.

The present inventors isolated and purified adseverin from bovine adrenal medulla and clarified its properties (Sakurai et al., Neuroscience 38:743-756, 1990; Sakurai et al., J. Biol. Chem. 226:4581-4584, 1991; Sakurai et al., J. Bio. Chem. 266:15979-15983, 1991).

Further, a hydrolyzed fragment of this protein was obtained and, based on the partial information of its amino acid sequence, oligonucleotide primers were synthesized. On the other hand, cDNA was prepared by reverse transcription from mRNA prepared from MDBK cells, a cell line established from bovine kidney (JCRB-Cell, obtained from Japan Foundation for Cancer Research). Then polymerase chain reaction (PCR) was performed with the use of the primers synthesized above to thereby specifically amplify the DNA fragment encoding bovine adseverin. Next, a cDNA library prepared from bovine adrenal medulla was screened by using the above-mentioned DNA fragment labeled with ^{32}P as a probe. From 3 overlapping clones thus obtained, the target gene encoding the actin filament-severing protein was assembled. Thus the entire base sequence of the gene was successfully identified.

Subsequently, the present inventors employed this bovine adseverin cDNA as a probe and screened a cDNA library prepared from human kidney mRNA by plaque hybridization under less stringent conditions. Thus they isolated human adseverin cDNA and successfully identified the entire base sequence of the same.

DISCLOSURE OF THE INVENTION

The present invention provides a gene encoding adseverin. More particularly, it provides a DNA containing a base sequence encoding the amino acid sequence represented by SEQ ID NO:4 or SEQ ID NO:6 in Sequence Listing, which optionally has partial replacement, deletion or addition, or a base sequence hybridizable therewith.

The present invention further provides a recombinant vector containing the gene encoding the adseverin protein.

The present invention furthermore provides prokaryotic or eukaryotic host cells transformed by the recombinant vector containing the gene encoding the adseverin protein.

The present invention furthermore provides a process for producing human adseverin protein which comprises incubating a transformant, which has been obtained via transformation by the recombinant vector containing the gene encoding the adseverin protein, and isolating and purifying the target protein thus produced.

The present invention furthermore provides the recombinant adseverin protein produced by the above-mentioned process.

The present invention furthermore provides an oligonucleotide hybridizable specifically with the gene encoding adseverin.

The present invention furthermore provides a method for regulating the formation of adseverin in an animal which comprises administering an oligonucleotide hybridizable specifically with the gene encoding adseverin to the animal.

The present invention furthermore provides an antibody capable of recognizing the adseverin protein.

By using a labeled adseverin cDNA fragment as a probe, the present inventors further performed *in situ* hybridization and studied the expression of adseverin mRNA in tissues to thereby clarify the distribution of adseverin in the tissues. Also, the actin-serving domain in adseverin was examined.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a photograph which shows the electrophoretic pattern of purified adseverin obtained from bovine adrenal medulla in comparison with purified gelsolin obtained from bovine aorta. SDS-PAGE was carried out by using 6.5 - 10.5% linear gradient gel. Lanes 1 and 2 show fractions from bovine aorta treated with a DNase I affinity column. Lane 1 corresponds to the EGTA eluate, while lane 2 corresponds to the 6 M urea eluate. Lanes 3 to 8 show fractions obtained from bovine adrenal medulla. Namely, lanes 3, 4, 5, 6, 7 and 8 correspond respectively to: the crude extract; the EGTA eluate of the DNase I affinity column; the 6 M urea eluate of the DNase I affinity column; the Q-Sepharose fraction containing adseverin; the Q-Sepharose fraction containing plasma gelsolin, cytoplasmic gelsolin and actin; and adseverin purified by HPLC gel filtration. Lane M shows molecular weight markers of 94,000, 67,000, 43,000 and 30,000 from top to bottom.

Fig. 2 shows a comparison between the partial amino acid sequence of an adseverin fragment of a molecular weight of 39,000 (C39) and the amino acid sequences of the corresponding parts of gelsolin and villin.

Fig. 3 shows the amino acid sequence of the N-terminus of a fragment obtained by digesting adseverin with thermolysin and the predicted location thereof in comparison with gelsolin.

Fig. 4 shows a restriction map of bovine adseverin cDNA. The bar designated as PCR stands for the cDNA produced by the reverse transcription from RNA of MDBK cells and PCR. The open bars numbered 19, 5 and 21 stand for individual cDNA clones

isolated from the λgt11 cDNA library of bovine adrenal medulla and employed in the construction of the adseverin cDNA.

Fig. 5 shows the amino acid sequence of bovine adseverin, which has been identified in the present invention, in comparison with the amino acid sequences of the corresponding segments of human gelsolin and human villin. The numbers at the right side designate the segment numbers for adseverin, gelsolin and villin. The largest homology resides between the segments 1 and 4, 2 and 5 and 3 and 6. The highly conserved motif sequences are shown in boxes. Putative polyphosphoinositide binding sites are boxed by dotted lines. The diagram with ellipses numbered 1 to 6 given below indicates 6 homologous segments of these proteins.

Fig. 6 is a photograph which shows the electrophoretic pattern of the expression of adseverin in *Escherichia coli* and purification thereof. In Fig. 6, A shows SDS-PAGE analysis of the expression of adseverin in *E. coli*. The transformant was incubated in the presence (lane 3) or absence (lane 2) of 0.4 mM IPTG for 3 hours. Then the pelleted cells were dissolved in an SDS sample buffer, heated and loaded onto an SDS-polyacrylamide gel. After electrophoresing, the gel was stained with Coomassie brilliant blue. The arrow indicates the adseverin band. Lane 1 shows molecular weight markers. In Fig. 6, B shows immunoblot analysis performed after the expression of adseverin in *E. coli* and purification of the same. The purified adseverin was separated with SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was stained with Ponceau S (lane 2) and, after destaining,

immunodetected with the use of an affinity purified antibody against adseverin (lane 3). Lane 1 shows molecular weight markers.

Fig. 7 shows the effects of adseverin expressed in *E. coli* on actin polymerization measured with a viscometer. Actin was polymerized in buffer P containing 0.1 mM of $CaCl_2$ (A) or 1 mM of EGTA (B). In Fig. 7, the data expressed in ○ and △ indicate the results of the polymerization in the presence of actin alone, while the data expressed in ● and ▲ indicate the results of the polymerization in the presence of the adseverin added at a molar ratio to actin of 1:30. The adseverin was added to the actin solution at a molar ratio of 1:30 at the points indicated by the arrows.

Fig. 8 provides light microscopic photographs, which show the morphology of organisms, of the expression of adseverin and its mRNA in the interface area between cortex and medulla of bovine adrenal gland. In each photograph, the upper part corresponds to the cortex while the lower part corresponds to the medulla. The sections were stained with Toluidine Blue (panel a) or successively with anti-adseverin rabbit antibody and fluorescein-conjugated anti-rabbit immunoglobulin (panels b and e). Panel d shows a phase-contrast image of the same field as the one of the panel e. Panels c and f show the images of *in situ* hybridization. The panels a to c are given in 120 x magnification, while the panels d to f are given in 280 x magnification.

Fig. 9 shows a comparison between the amino acid sequence of human adseverin and the amino acid sequence of bovine

adseverin. In Fig. 9, the upper and lower columns correspond respectively to the human amino acid sequence and the bovine amino acid sequence. These amino acid sequences are completely identical with each other at the amino acids with the mark * and highly analogous at the amino acids with the mark ·. Putative phospholipid binding sites are boxed by solid lines.

DETAILED DESCRIPTION OF THE INVENTION

cDNA encoding adseverin can be obtained by, for example, preparing mRNA from adseverin-producing cells and then converting it into a double stranded cDNA by a known method.

In the present invention, mRNA of the bovine adseverin are obtained from MDBK cells, which is a cell line established from bovine kidney, and bovine adrenal medulla (Madin et al., Proc. Soc. Exp. Biol. 98:574-576, 1958), while mRNA of the human adseverin is obtained from human kidney mRNA purchased from CLONTECH Laboratories Inc. However, the mRNA sources are not restricted thereto but use can be made of adrenal medulla chromaffin cells, kidney medulla, thyroid tissue homogenizate, etc. therefor.

The RNA may be prepared in accordance with, for example, the method of Chirgwin et al. (Biochemistry 18:5294-5299, 1979). Namely, the whole RNA can be obtained by treating the RNA source with guanidine thiocyanate followed by cesium chloride gradient centrifugation. Alternatively, use can be also made of methods employed for cloning genes of other physiologically active proteins, for example, treatment with a surfactant or phenol in the presence of a ribonuclease inhibitor (for example,

a vanadium complex).

To obtain the double stranded cDNA from the mRNA thus obtained, reverse transcription is performed by, for example, using the mRNA as a template and an oligo (dT) or random primer, which is complementary to the poly A-chain at the 3'-end, or an synthetic oligonucleotide, which corresponds to a part of the amino acid sequence of adseverin, as a primer so as to synthesize a DNA (cDNA) complementary to the mRNA.

In the present invention, the bovine adseverin cDNA is obtained in the following manner. Namely, reverse transcription is carried out by using random hexamers as primers. Next, the resulting product is amplified by PCR with the use of condensed primers to give a PCR product corresponding to a partial cDNA of adseverin of about 700 bp. Then this PCR product is subcloned into pBluescript SK(-) (Stratagene). Next, A λgt11 cDNA library prepared from bovine adrenal medulla is screened with the use of the ^{32}P-labeled cloned PCR product as a probe. In the present invention, 3 plaques are thus obtained and the target cDNA encoding adseverin is assembled on the basis of the overlapping base sequence of these plaques. Thus it is found out that the open reading frame is a protein of 80527 dalton composed of 715 amino acids (see SEQ ID NO: 4 in Sequence Listing).

The cDNA of human adseverin is obtained in the following manner. That is, a double stranded cDNA is synthesized by using TimeSaver™ cDNA Synthesis Kit (Pharmacia).

Then the double stranded cDNA thus synthesized is fractionated in size by using Spun Column included in the

above-mentioned Kit or agarose electrophoresis. Thus a cDNA of about 400 bp or more (in the former case) or about 2 to 3 kbp (in the latter case) is taken up exclusively. After ligating an adaptor to one end, the cDNA is integrated into a vector. Then the cDNA thus integrated into the vector is subjected to packaging with the use of GIGAPACK® II PACKAGING EXTRACT (STRATAGENE) to give a cDNA library.

Next, the cDNA library is screened under less stringent conditions by using thermally denatured bovine adseverin cDNA as a probe. Thus one positive phage clone is obtained. Then its cDNA moiety is amplified by PCR and integrated into a plasmid vector to thereby give a clone pADa-17. When partly sequenced, the base sequence of this clone shows a very high homology (80 - 90%) with the base sequence of the bovine adseverin cDNA. In contrast, it shows only a low homology of 60% or below with gelsolin which is a protein belonging to the adseverin family and having a known base sequence, suggesting that this is a gene obviously different therefrom. Thus it is assumed that this clone is human counter part of adseverin. However, this clone is about 1 kbp in full length and thus seemingly fails to contain the entire coding region. Accordingly, further screening should be carried out.

Thus plaque hybridization is carried out by using the above-mentioned clone pADa-17 as a probe under usual conditions with an elevated strictness. In this step, use is made of a library newly prepared from human kidney mRNA by concentrating cDNAs of 2 to 3 kbp exclusively in order to efficiently obtain clones of the full length. Thus 5 positive phage clones are

obtained therefrom and excised into a plasmid [pBluecript^{R} SK(-) vector] with ExAssist™/SOLR SYSTEM to thereby give plasmid clones phAD-2 to 6. Among these plasmid clones, the base sequences of phAD-2 and phAD-4 are identified. By combining these base sequences, a sequence represented by SEQ ID NO:5 in Sequence Listing is determined. From this base sequence, an open reading frame composed of 715 amino acids and having ATG at the 79-position as the initiation codon (Met) is found out. Fig. 9 shows the result of a comparison of this amino acid sequence with the bovine adseverin amino acid sequence. These amino acid sequences show a homology of about 92% at the amino acid level, which suggests that this protein has been very well conserved beyond difference in species. It is also clarified that these amino acid sequences are highly analogous in many amino acids, even though they are not completely the same as each other. Although a high homology of about 90% is observed at the base level, the homology shows a rapid decrease after the stop codon, which seemingly reflects the difference in species.

In Fig. 9, putative phospholipid binding sites are boxed by solid lines. The putative phospholipid binding sites in bovine adseverin, namely, (112)KGGLKYKA(119) and (138)RLLHVKGRR(146) are both completely conserved in human adseverin too. Thus it is suggested that the difference in sensitivity to phospholipids between adseverin and gelsolin might be caused by the difference in the amino acid sequences of these regions. It is reported that adseverin is located in cells in the vicinity of cell membrane. Thus, the regulation

of the adseverin activity by cell membrane constituents, if any, might be highly important. Since gelsolin is also activated by Ca^{2+}, there is a fair possibility that phospholipids would control how to utilize these proteins case by case.

By using the cloned gene of the present invention encoding adseverin thus obtained, adseverin can be produced in a large amount by gene recombination techniques and used for medicinal purposes.

Accordingly, prokaryotic or eukaryotic host cells can be transformed by appropriate vectors into which the gene of the present invention encoding adseverin has been integrated.

Further, the gene can be expressed in each host cell by introducing an adequate promoter or a sequence relating to the expression into these vectors. Moreover, the target gene may be ligated to another gene encoding a polypeptide and expressed as a fused protein to thereby facilitate purification or elevate the expression dose. It is also possible to excise the target protein by effecting adequate treatments in the purification step.

It is generally considered that an eukaryotic gene shows polymorphism as known in the case of human interferon gene. In some cases, one or more amino acids are replaced due to this polymorphism, while changes occur not in amino acids but exclusively in base sequence in other cases.

It is sometimes observed that a polypeptide having the amino acid sequence of SEQ ID NO: 4 or 5 in Sequence Listing having the deletion, addition or replacement of one or more

amino acids shows an actin filament-severing activity. For example, it is publicly known that a polypeptide, which is obtained by replacing a base sequence corresponding to cysteine of human interleukin 2 (IL-2) by another base sequence corresponding to serine, sustains the IL-2 activity (Wang et al., Science 224:1431, 1984). Thus the techniques for constructing the variants of these genes encoding adseverin are well known by those skilled in the art.

Moreover, bovine adseverin is highly homologous with human adseverin and highly analogous in many amino acids even though they are not completely the same, as described above. Accordingly, genes having partial replacements of bovine or human adseverin and chimeric genes thereof also fall within the scope of the present invention.

When adseverin is expressed in eukaryotic cells, sugar chain(s) are frequently added thereto and the addition of the sugar chains can be controlled by converting one or more amino acids. In such a case, the expression product sometimes has an actin filament-severing activity. Therefore, the present invention includes any gene which is obtained by artificially varying the gene encoding human adseverin and encodes a polypeptide, so long as the obtained polypeptide has an actin filament-severing activity.

Furthermore, the present invention includes a gene which is capable of giving a polypeptide having an actin filament-serving activity and hybridizable with a gene represented by SEQ ID NO:4 or 5 in Sequence Listing. The hybridization may be carried out under the conditions commonly

employed in probe hybridization (see, for example, Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989).

An expression vector may contain a replication origin, a selective marker, a promoter, an RNA splicing site, a polyadenylation signal, etc.

Examples of the prokaryotic cells to be used as the host cells in the expression system include *E. coli* and *Bacillus subtilis*. Examples of the eukaryotic cells usable as the host cells include yeasts and Myxomycota. Alternatively, insect cells such as Sf9 may be used as the host cells. In addition, use can be made of host cells with an animal origin such as COS cells and CHO cells therefor.

The protein, which has been produced by incubating a transformant transformed by the gene encoding adseverin, can be purified either in the cells or after isolating from the cells.

Adseverin may be isolated and purified by procedures commonly employed in the isolation and purification of proteins. For example, various chromatographies, ultrafiltration, salting out, dialysis, etc. may be adequately selected and combined therefor.

According to the present invention, an antisense DNA can be prepared on the basis of the base sequence of the gene encoding adseverin. The antisense DNA, which has a base sequence complementary to the mRNA, forms base pairs with the mRNA and blocks the transmission of genetic information, thus regulating the synthesis of the adseverin protein, i.e., the

final product. The antisense DNA usable in the present invention is an oligonucleotide hybridizable specifically with a base sequence which encodes the amino acid sequence represented by the SEQ ID NO:4 or 5 in Sequence Listing.

The term "oligonucleotide" as used herein means an oligonucleotide composed of a base occurring in nature with a sugar moiety binding thereto via a phosphodiester bond of the inherent meaning or its analogue. That is to say, the first group meant thereby includes natural oligonucleotides and synthetic oligonucleotides prepared from subunits occurring in nature or homologues thereof. The term "subunit" means a combination of a base with a sugar binding to the adjacent subunit via a phosphodiester bond or another bond. The second group of the oligonucleotide includes analogues of the above-mentioned oligonucleotides taking the same roles as oligonucleotides but having residues containing some parts which are not observed in nature. Oligonucleotides, which have been chemically modified at the phosphate group, the sugar moiety, or the 3'- or 5'-end to enhance the stability, also fall within this category. Examples thereof include oligophosphorothioate and oligomethylphosphonate wherein an oxygen atom in the phosphodiester bond between nucleotides has been replaced respectively by a sulfur atom and $-CH_3$. The phosphodiester bond may be replaced by another structure which is nonionic and nonchiraric. As oligonucleotide analogues, use can be made of those containing modified bases, i.e., purine and pyrimidine which are not observed in nature.

The oligonucleotide to be used in the present invention preferably has 8 to 40, still preferably 15 to 30, subunits.

It is preferable in the present invention that the target part of mRNA, with which the oligonucleotide is hybridized, is the transcription initiation site, the translation initiation site, the intron/exon junction or the 5'-capping site. It is required to select a site free from any strict hindrance by taking the secondary structure of the mRNA into consideration.

The oligonucleotide of the present invention may be prepared by synthesis methods publicly known in the art, for example, the solid phase synthesis with the use of a synthesizer manufactured by Applied Biosystems, etc. It is also possible to prepare other oligonucleotide analogues such as phosphorothioate or alkylated derivatives by using similar methods [Murakami et al., "Kinosei Antisense DNA no Kagaku Gosei (Chemical Synthesis of Functional Antisense DNA)", Yuki Gosei Kagaku (Organic Synthesis Chemistry), 48 (3):180-193, 1990].

By administering an oligonucleotide hybridizable specifically with the gene of the present invention encoding adseverin to an animal, the formation of adseverin in the animal can be regulated. As described above, adseverin might relate to the multiplication of blood vessel smooth muscles. The multiplication of blood vessel smooth muscles is regarded as one of the factors causing angiostenosis in blood vessel transplantation in bypass operation, etc. or restenosis which is observed at a ratio of 30 to 40% after PTCA. Accordingly,

the antisense DNA of the gene encoding adseverin, the administration of which can suppress the multiplication of blood vessel smooth muscles, is usable as a preventive and remedy for these stenoses. For example, it is expected that angiostenosis can be prevented by soaking the blood vessel to be transplanted in a solution containing the oligonucleotide of the present invention to thereby incorporate the oligonucleotide into the cells followed by the transplantation. It is also possible to prevent restenosis by administering the oligonucleotide of the present invention with the use of a PTCA catheter or stent.

An antibody of the present invention capable of recognizing the adseverin protein can be constructed in accordance with a conventional method [see, for example, Shinseikagaku Jikken Koza (New Biochemistry Experiment Lecture) 1, Tanpakushitsu (Protein) I, 389-397, 1992] by immunizing an animal with adseverin serving as the antigen and collecting and purifying the antibody thus produced in the animal body. The anti-adseverin antibody thus obtained is usable in various immunological assays such as enzyme immunoassays (for example, ELISA), radioimmunoassays and immunofluorescent techniques.

EXAMPLES

To further illustrate the method for obtaining the gene of the present invention encoding adseverin and the expression of this gene in host cells in greater detail, the following Examples will be given. However, it is to be understood that the present invention is not restricted thereto.

Example 1: Isolation and purification of bovine adseverin

Bovine adrenal glands were obtained from a slaughterhouse. All the procedures described below were carried out at 4°C. The adrenal medullae were carefully separated from cortices and minced with scissors. 80 g of the material thus obtained was homogenized in thrice by volume as much buffer A (pH 8.0) containing 40 mM of Tris-HCl, 4 mM of EGTA, 2 mM of EDTA, 1 mM of DTT, 1 mM of DFP, 1 mM of PMSF, 10^{-6} M of E-64-c, 10 μg/ml of aprotinin (Trasylol, Bayer) and 0.02% of NaN_3 in a Waring blender. The homogenate was centrifuged at 13,000 g at the maximum for 30 minutes. The supernatant was filtered and further centrifuged at 150,000 g at the maximum for 90 minutes. To the supernatant were added 1 mol solutions of $CaCl_2$ and $MgCl_2$ to give final concentrations of 0.5 and 1 mM respectively. Then the resulting solution was passed through a DNaseI-Affi-Gel 15 column which had been equilibrated with buffer B (pH 7.5) containing 50 mM of KCl, 20 mM of Tris-HCl, 0.5 mM of $CaCl_2$, 1 mM of $MgCl_2$, 0.1 mM of PMSF and 0.02% of NaN_3. Then the column was washed successively with the buffer B and the modified buffer B containing not 50 mM but 0.6 M of KCl.

Next, Ca^{2+}-sensitive proteins were eluted with the modified buffer B containing 10 mM of EGTA as a substitute for 0.5 mM of $CaCl_2$ and eluted with the modified buffer B containing 6 M of urea. Thus 3 Ca^{2+}-sensitive actin-binding proteins and actin (molecular weight: 42,000) were eluted with the EGTA-containing buffer. The results of SDS PAGE suggested that these 3 proteins had molecular weights of 86,000, 84,000 and

74,000 respectively (Fig. 1, lanes 1 to 4). The column was regenerated by washing with the buffer B and stored at 4°C.

The EGTA eluate thus collected was adjusted to pH 8.2 with 1 M Tris and then applied to a Q-Sepharose ion exchange column (1.5×12 cm) which had been equilibrated with a solution (pH 8.2) containing 50 mM of KCl, 20 mM of Tris-HCl, 1 mM of EGTA, 0.1 mM of PMSF, 7 mM of 2-mercaptoethanol and 0.02% of NaN_3. Proteins were eluted with a linear KCl gradient from 50 to 250 mM and then with 1 M KCl. The first peak fraction corresponding to 0 to 150 mM KCl contained the protein of a molecular weight of 74,000 together with a small amount of contaminating proteins (Fig. 1, lane 6). The proteins of molecular weights of 86,000 and 84,000 and actin were contained in the second peak which was the eluate with 1 M KCl (Fig. 1, lane 7).

The fraction containing the protein of a molecular weight of 74,000 was collected, concentrated and applied to a gel filtration HPLC column (TSK-G3000SW, Tosoh) which had been equilibrated with buffer C (pH 7.0) containing 150 mM of NaCl, 20 mM of Tris-HCl, 1 mM of EGTA, 0.1 mM of DTT and 0.02% of NaN_3 (Fig. 1, lane 8). The peak fractions were collected and stored on ice.

Example 2: Protease digestion of bovine adseverin

(1) Digestion by *Staphylococcus* V8 protease

Adseverin in digestion buffer C (1 mM of EGTA, 1 mM of DTT, 0.02% of NaN_3 and 50 mM of NH_4HCO_3) was digested by *Staphylococcus* V8 protease at room temperature at a ratio of 1:25 (wt/wt). The reaction was stopped by adding 1 mM of DFP

followed by SDS-PAGE analysis. Thus it was found out that adseverin was digested into two major fragments of 42,000 and 39,000 in molecular weight. After digesting by the V8 protease over a prolonged period, the fragment of 39,000 in molecular weight was further digested into fragments of molecular weights of 28,000 and 15,000, while the fragment of 42,000 in molecular weight remained stable.

(2) Digestion by trypsin

Adseverin in buffer D (1 mM of EGTA, 1 mM of DTT, 0.02% of NaN_3 and 20 mM of Tris-HCl, pH 8.0) was digested by trypsin at a ratio of 1:200. After reacting at 25°C for 60 minutes, a 200 mM solution of PMSF in ethanol was added to give a final PMSF concentration of 4 mM followed by SDS-PAGE analysis. Thus it was found out that adseverin was also digested into two fragments of 42,000 and 39,000 in molecular weight and no further digestion occurred thereafter.

From the results of recognition reactions of 2 antigelsolin polyclonal antibodies with the above-mentioned 2 fragments, it was confirmed that the fragment of 39,000 in molecular weight was not a digestion product of the fragment of 42,000 in molecular weight.

(3) Purification of V8 protease digestion product

The V8-digestion product was applied to an HPLC DEAE ion exchange column (DEAE-SPW, Tosoh) which had been equilibrated with buffer D. The fragment of 39,000 in molecular weight was adsorbed by the column, while the one of 42,000 in molecular weight was eluted with an NaCl gradient of 0 to 150 mM and obtained as a single peak at the NaCl

concentration of 10 mM. Next, the buffer D containing no EGTA but 0.5 mM $CaCl_2$ was used. Thus the fragment of 39,000 in molecular weight was eluted but the fragment of 42,000 in molecular weight was recovered only in a small amount. These 2 V8 protease-digestion fragments thus purified showed almost the same patterns in SDS-PAGE.

(4) Identification of N-terminal amino acid sequence

The N-terminal amino acid sequences of 2 fragments purified in the above (3) and native adseverin were discussed. Although the N-termini of native adseverin and the fragment of 42,000 in molecular weight were blocked, it was clarified by the Edman degradation method that the vicinity of the N-terminus of the fragment of 39,000 in molecular weight had the following amino acid sequence of SEQ ID NO:1 in Sequence Listing:

KVAHVKQIPFDA.

This sequence was compared with those of publicly known actin filament-serving proteins gelsolin (Kwiatkowski et al., Nature 323:455-458, 1986) and villin (Bazari et al., Proc. Natl. Acad. Sci. U.S.A. 85:4986-4990, 1988). As a result, the above-mentioned sequence was similar to the hinge region located between the conserved repetition segments 3 and 4 in gelsolin and villin, i.e., the middles of these molecules, as shown in Fig. 2. Thus, it is suggested that the fragment of 42,000 in molecular weight is a protein located in the NH_2-terminal half of adseverin (hereinafter referred to as "N42"), while the fragment of 39,000 in molecular weight is a protein located in the COOH-terminal half of adseverin (hereinafter referred

to as "C39").

(5) Actin-binding properties of N42 and C39

The actin-binding properties of N42 and C39 obtained above were examined by using an actin monomer (G-actin) bound to agarose beads. As a result, it was clarified that N42 and C39 both bound to G-actin in the presence of calcium but not in the absence of calcium.

(6) Identification of functional domain of adseverin (digestion of N42 by thermolysin)

When N42 was digested by thermolysin which was a metaproteinase, 5 fragments including those of 31,000, 30,000 and 16,000 in molecular weight and 2 different ones of 15,000 in molecular weight were obtained. These fragments were purified by HPLC. The fragments of 31,000 and 30,000 in molecular weight were named respectively TL1 and TL2, while the other 3 fragments were named TL3 (molecular weight: 16,000), TL4 (molecular weight: 16,000) and TL5 (molecular weight: 15,000) in the order of elution from the HPLC column. The N-termini of TL1 and TL3 were not detected by an antibody A, since they were blocked as in the case of N42 and native adseverin. On the other hand, TL2 and TL5 reacted with the antibody A. Based on these results, it is estimated that N42 has 2 cleavage sites with the mapping of the fragment as shown in Fig. 3.

The amino acid sequences of TL4 and TL5, the N-termini of which were not blacked, were analyzed by the Edman degradation method. As a result, it is proved that the N-terminal amino acid sequence of TL4 is the following

one represented by SEQ ID NO:2 of Sequence Listing:

VLTNDLTAQ

which is homologous with the sequence of the hinge region between the segments 1 and 2 of gelsolin. On the other hand, the N-terminal amino acid sequence of TL5 is the following one represented by SEQ ID NO:3 of Sequence Listing:

ITNRK

which is homologous with the sequence of the hinge region between the segments 2 and 3 of gelsolin (Fig. 3).

Accordingly, it is considered that adseverin has a structure similar to that of gelsolin. Similar to gelsolin, the N-terminal half of adseverin is composed of 3 repetition segments each corresponding to a protein digestion fragment of up to 15 kDa.

Example 3: Synthesis of degenerate primers

Mix primers, which contained all codons potentially serving as genes encoding the N-terminal amino acid sequence of the second segment (S2) of N42 identified in Example 2 and the N-terminal amino acid sequence of C39, were synthesized by using an Applied Biosystems 380B DNA synthesizer. To the 5' ends of the sense and antisense primers, BamHI site and ClaI site were added respectively.

The sequences of the degenerate primers were as follows:

5' . . . GATGCGGATCCAA(C/T)GA(C/T)(C/T)T(A/C/G/T)AC(A/C/G/T)GC(A/C/G/T)CA . . . 3'; and

5' . . . GATGCATCGATAC(A/G)TG(A/C/G/T)GC(A/C/G/T)AC(C/T)TT(C/T)TC . . . 3'.

Example 4: Reverse transcription and PCR

RNA was prepared in accordance with the method of Chirgwin et al. (Biochemistry 18:5294-5299, 1979) from MDBK cells, i.e., a cell line established from bovine kidney (JCRB-Cell, obtained from Japan Foundation for Cancer Research: Madin et al., Proc. Soc. Exp. Biol. Med. 98:574-576, 1958).

Reverse transcription and PCR were carried out in accordance with the method of Kawasaki [in PCR protocols: A guide to Methods and Application (Innis et al. eds) pp. 21-27, Academic Press, San Diego, 1990]. Random hexamers (Pharmacia) were employed for the reverse transcription, while the degenerate primers obtained in Example 3 were employed for PCR [Lee et al., in PCR protocols: Guide to Methods and Application (Innis et al. eds) pp. 46-53, Academic Press, San Diego, 1990]. PCR was effected first in 5 cycles each consisting of 1 minute at 94°C, 1 minute at 37°C and 2 minutes at 72°C, wherein the treating temperature was slowly elevated from 37 to 72°C for 2.5 minutes. Next, 29 cycles each consisting of 1 minute at 94°C, 1 minute at 50°C and 2 minutes at 72°C were repeated in a usual manner followed by 1 cycle consisting of 1 minute at 94°C, 1 minute at 50°C and 10 minutes at 72°C. Then the reaction mixture was allowed to stand at 4°C.

Example 5: Cloning of PCR product

The PCR product obtained in Example 4 was electrophoresed on a 1% agarose gel containing 1 μg/ml of ethidium bromide. As a result, the main band was observed at about 700 bp. Then it was excised from the gel and purified

with the use of a GENECLEAN II Kit (BIO 101 Inc.). Its size could be estimated depending on the locations of the fragments from which the degenerate primers were derived, on the basis of an assumption that adseverin might be highly homologous with gelsolin in the primary structure. The product thus purified was digested with BamHI and ClaI and cloned into pBluescript SK(-) (Stratagene).

When the cloned PCR product was sequenced, a nucleotide sequence encoding the N-terminus of the third segment (S3) of N42 was contained therein. Thus it was confirmed that this PCR product actually corresponded to a part of the adseverin cDNA. The high homology (identity at nucleotide level: 64%) between this sequence and the human gelsolin sequence also supported this idea.

The PCR product thus obtained was ^{32}P-labeled and employed as a probe in the subsequent screening.

Example 6: Library screening

A λgt11 cDNA library prepared from bovine adrenal medulla (CLONETECH) was screened in accordance with the standard method (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) with the use of the ^{32}P-labeled PCR product obtained in Example 5 which represented the partial cDNA of adseverin. After screening twice, well-isolated positive plaques were taken out and phages in each plaque were released into 200 μl of distilled water and incubated at room temperature for 1 hour. Then the phage solution was frozen, thawed and heated at 90°C for 10 minutes.

By using an appropriate amount of the phage solution as a template, the insert of the recombinant phage DNA was amplified by PCR with the use of a pair of primers which contained sequences from the upstream and downstream of the EcoRI-specific site of λgt11. PCR was carried out under the same conditions as those described in Example 4. To the 5'-ends of these primers, XhoI site and NotI site were respectively added. One of the primers had the following sequence:

5' . . . AdseverinCTCGAGGGTGGCGACGACTCC . . . 3'; and another one had the following sequence:

5' . . . AdseverinGCGGCCGCTTGACACCAGACCAA . . . 3'.

After the completion of PCR, the reaction product was electrophoresed on a 1% agarose gel. The amplified insert DNA was excised and purified by using a GENECLEAN II kit. After digesting with XhoI and NotI, the insert cDNA was cloned into pBluescript SK(-) which had been digested with XhoI and NotI.

By using the cloned PCR product as a probe, the cDNA library of bovine adrenal medulla was screened. Thus 3 overlapping cDNA clones were plaque-purified from 2×10^6 recombinant phages.

The above-mentioned 3 cDNA clones overlapping each other are shown by Nos. 19, 5 and 21 in Fig. 4. The base sequences of these cloned DNAs were examined in both directions by the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci. U.S.A., 74:5463-5467, 1977) and the entire nucleotide sequence of adseverin was identified based thereon. This nucleotide sequence is represented by SEQ ID NO:4 in Sequence Listing. Fig. 4 shows a restriction map of the cDNA

thus assembled.

The nucleotide sequence of the assembled cDNA and the amino acid sequence corresponding to the longest open reading frame are also represented by SEQ ID NO:4 in Sequence Listing. The open reading frame encodes a protein of 80527 dalton, consisting of 715 amino acids. The first ATG is located on 27 nucleotides 3'-side to the start of the clone and represents a good vertebrae translation initiation consensus sequence. A comparison of the adseverin cDNA sequence with the sequences of gelsolin and villin also supports that the ATG represents the initiation codon and that the assembled cDNA contains the entire coding sequence of adseverin.

Next, a cDNA of 2418 bp which contained the entire coding region of adseverin was assembled from the 3 overlapping clones with the use of AccI and HindIII sites. This cDNA was integrated into the XhoI and NotI sites of pBluescript SK(-) to thereby give pSK-adseverin.

Example 7: Comparison of predicted amino acid sequence of adseverin with amino acid sequences of human gelsolin and villin

Biochemical analyses and the predicted amino acid sequence from cDNA have revealed that human gelsolin and villin each consists of 6 homologous segments (Bazari et al., Proc. Natl. Acad. Sci. U.S.A. 85:4986-4990, 1988; Matsudaira et al., Cell 54:139-140, 1988; Way et al., J. Mol. Biol. 203:1127-1133, 1988). The segments 1, 2 and 3 have higher homologies respectively with the segments 4, 5 and 6 than any other combinations. The analysis on the predicted amino acid

sequence of adseverin has revealed that adseverin has 6 homologous segments too. The segments 1 to 6 have homologies respectively with the corresponding segments of gelsolin and villin (Fig. 5). As Fig. 5 clearly shows, motifs B, A and C existing in each of the 6 segments of gelsolin and villin were also found out in the 6 segments of adseverin. These facts indicate that adseverin belongs to gelsolin family proteins.

Moreover, the putative polyphosphoinositide binding sequences existing in gelsolin and villin were also found in adseverin in the regions corresponding to the regions of gelsolin and villin, i.e., the first and second segments (S1, S2). This fact agrees with the data that the protein fragment-severing activity corresponding to S1-2 of adseverin was inhibited by polyphosphoinositide. These sequences are boxed in Fig. 5 and shown as a model view in Table 1. One of these 2 putative sequences completely agreed with the consensus sequence, while another one located in the first segment was different from the consensus sequence only in one amino acid. That is to say, it had alanine at the COOH-terminal while the consensus sequence had a basic amino acid at this position. Thus this domain of adseverin had a less basic nature than that of the corresponding domain of gelsolin. This difference could partly account that acidic phospholipids other than phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate, for example, phosphatidylinositol and phosphatidylserine can inhibit the serving activity of adseverin but not that of gelsolin.

Table 1
Predicted polyphosphoinositide
binding sites of adseverin in comparison
with other actin filament-severing proteins

Protein	Location of binding site	Amino acid sequence
adseverin	112 - 119	KGG-LKYKA
gelsolin	135 - 142	KSG-LKYKK
villin	112 - 119	KQG-LVIRK
adseverin	138 - 146	PLLHVKGRR
gelsolin	161 - 169	RLFQVKGRR
villin	138 - 146	RLLHVKGKR
consensus		K KK XX(X)XKX R RR

Example 8: Expression of adseverin cDNA in *E. coli*

The bovine adseverin cDNA (pSK-adseverin) obtained in Example 6 was amplified by PCR. Primers employed in PCR were so designed that the initiation codon (ATG) of the product cDNA constituted a part of NdeI while the termination codon (TAA) was immediately followed by the XhoI site. The cDNA thus obtained was integrated into an expression vector pET-23a (Novagen) via the NdeI and XhoI sites. The resulting recombinant vector pET-adseverin was then introduced into competent BL21(DE3)pLysS cells by the method of Chung et al. (Proc. Natl. Acad. Sci. U.S.A. 86:2172-2175, 1988). Transformants were selected, incubated and induced with IPTG (isopropyl-β-thiogalactopyranoside) in accordance with the method of Studier et al. [in Methods in Enzymology, Gene Expression Technology (Goeddle eds.) Vo., 185, pp. 60-89, Academic Press, San Diego, 1991]. Namely, a colony resistant against ampicillin and chloramphenicol was picked up and incubated in M9ZB medium supplemented with 50 μ g/ml of ampicillin. When the expression of the cDNA was induced by IPTG, a protein of approximately 74 kDa on SDS-PAGE was produced (Fig. 6A, indicated by arrow). In contrast, the untransformed control BL21(DE3)pLysS produced no extra protein on the induction with IPTG. The size (i.e., 74 kDa) of the induced protein on SDS-PAGE was the same as that of adseverin prepared from bovine adrenal medulla.

The culture supernatant of the transformed *E. coli* was purified by substantially the same methods the one employed for the isolation and purification of adseverin from

bovine adrenal medulla in Example 1. The purified protein was electrophoresed on SDS-PAGE and transferred onto a nitrocellulose membrane. When reacted with an antibody specific to adseverin, this protein underwent an immunological reaction with this protein, as shown in Fig. 6B. Based on the apparent size of this protein on SDS-PAGE and its immunoreactivity with the adseverin specific antibody, it was confirmed that this protein was the cDNA encoding adseverin.

Example 9: Actin filament-severing activity of adseverin produced by *E. coli*

To examine whether or not the adseverin produced by *E. coli* had a Ca^{2+}-dependent actin filament-severing activity similar to native adseverin, effects of the adseverin on actin polymerization were measured with a viscometer.

0.15 mg/ml of actin was polymerized in buffer P (50 mM KCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl, pH 7.2) with 1 mM of EGTA or 0.1 mM of $CaCl_2$ at 25.5°C in the presence or absence of adseverin at a molar ratio to actin of 1:30.

As Fig. 7 shows, the viscosity of the actin solution was affected by adseverin exclusively in the presence of Ca^{2+} (compare Fig. 7A with 7B). In the presence of Ca^{2+}, adseverin promoted the nucleation in the process of actin polymerization so as to lower the final viscosity of the polymerized actin solution. When adseverin was added to the polymerized actin solution (indicated by arrows), the specific viscosity showed a sudden drop in the case of the solution containing Ca^{2+}.

These results were substantially the same as those obtained by using adseverin prepared from bovine adrenal

medulla , which indicated that the protein produced by the gene recombination techniques according to the present invention had an actin filament-severing activity similar to native adseverin.

Example 10: *In situ* hybridization

A 329 bp fragment of the bovine adseverin cDNA (#2090 - #2418) was labeled with digoxigenin-dUTP by using a DIG DNA labeling and detection kit (Boehringer Mannheim).

The part of fresh bovine adrenal gland containing the interface region between cortex and medulla was fixed with 1% paraformaldehyde in phosphate saline buffer (PBS) in the slaughterhouse. In the laboratory, it was cut into small pieces and washed with PBS. Next, the samples were immersed stepwise in 8, 12, 16 and 20% sucrose-PBS for 24 hours. Then the samples were embedded in TISSUE-TEM (Miles Sceitific) and frozen in liquid nitrogen. The frozen samples were cut into sections of 5 to 7 μm with a microtome and collected on a slide glass.

Some of these sections were stained with 0.5% of Toluidine Blue in PBS and 50% of glycerol in PBS and stored in this solution.

For immunofluorescent staining, the sections were fixed with 1% paraformaldehyde-PBS for 1 minute and with acetone for 5 minutes. After treating with 1% of Triton X-100 in PBS and washing with PBS, the sections were introduced into a blocking solution containing 2.5% of bovine serum albumin and 2.5% of chick serum in PBS and incubated together with anti-adseverin antibody (method for the preparation of the anti-adseverin

antibody will be described in Example 18 hereinafter) in the blocking solution at 37°C for 3 hours. Then the sections were washed successively with a solution containing 400 mM of $MgCl_2$ and 20 mM of Tris-HCl (pH 8.6) and PBS. Then they were incubated together with FITC-conjugated anti-rabbit IgG in the blocking solution at 37°C for 1 hour. After thoroughly washing by the same procedure with the use of the same solutions as those described above, the sections were embedded in PBS containing 50% of glycerol and 2.5% of 1,4-diazabicyclo[2,2,2]octane (Wako Chemical Co., Ltd.) and observed under a Nikon FEX-A fluorescent microscope.

For *in situ* hybridization, the sections were incubated in double strength standard saline citrate (2×SSC, 1×SSC = 0.15 M NaCl, 15 mM Na-citrate, pH 7.0) for 10 minutes at room temperature and then in a pre-hybridization solution (5×SSC, 50% formamide, 0.1% Tween 20, 50 μg/ml heparin, 100 g/ml sonicated and denatured salmon sperm DNA) at room temperature for 1 hour.

After removing the pre-hybridization buffer, a fresh pre-hybridization buffer containing 0.5 μg/ml of the digoxigenin-labeled DNA probe was applied to the sections. Then the sections were covered with glass coverslips which were next sealed with rubber cement.

The DNA probe was denatured in an oven at 80°C for 10 minutes followed by incubation in the oven at 42°C overnight. Then the coverslips were removed by using a glass cutter and the sections were washed successively with 2×SSC at room temperature for 30 minutes, 0.1×SSC at 42°C for 30 minutes and

2×SSC at room temperature for 15 minutes.

The probes in the sections were detected by using a DIG DNA labeling and detection kit (Boehringer Mannheim). Then the sections incubated together with the digoxigenin-labeled DNA probe were washed in a washing buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature for 10 minutes, then incubated together with 0.5% (w/v) of Boehringer blocking reagent in the washing buffer and finally washed with the washing buffer.

Subsequently, the sections were incubated together with alkaline phosphatase-conjugated anti-digoxigenin antibody (150 mU/ml) at 37°C in the dark for 2 hours. After washing with the washing buffer twice, the slides were briefly treated with a solution containing 100 mM of Tris-HCl, 100 mM of NaCl and 20 mM of $MgCl_2$ (pH 9.5) and incubated together with the same solution containing nitro blue tetrazolium salt, 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml of levamisole at room temperature in the dark for 3 hours. The color development was stopped by using 10 mM of Tris-HCl and 1 mM of EDTA (pH 8.0).

The sections kept in glycerol were observed under a light microscope.

At a low magnification, the color development was observed in the medulla but not in the cortex except in the area adjacent to the medulla. Next, the interface area between the medulla and the cortex was observed at higher magnifications. Toluidine Blue staining (Fig. 8a) revealed that the cells in the cortex were tightly packed, whereas the

cells in the medulla were loosely distributed and classified into groups by sheath-like structures containing vessels. The cortex and the medulla were easily distinguishable from each other in both of the *in situ* hybridization and the immunofluorescent staining depending on the cellular characteristics as described above without effecting counter-staining. Fig. 8c and f show the results of the *in situ* hybridization observed at middle and high magnifications respectively. Staining was observed mainly in loosely packed cells corresponding to the medullary chromaffin cells. In addition, a small number of cells in the cortex facing the medulla were also stained as shown by arrows.

The adseverin distribution of the same pattern was observed in the immunofluorescent staining with the anti-adseverin antibody (Fig. 8b and e). Namely, fluorescence was observed in the chromaffin cells of the medulla and in the cells in the cortex facing the medulla. In the chromaffin cells, fluorescence was mainly observed in the subplasmalemmal region.

In summary, it was demonstrated that the adseverin mRNA and the adseverin protein were both expressed in the adrenal medulla but not in most part of the cortex. Exceptionally, the expression of both of the adseverin mRNA and the adseverin protein was observed in a part of the cortex facing the medulla. Thus it is concluded that such differential expression of adseverin in the parts of bovine adrenal gland is controlled at the transcription level. Secretion in the mode of exocytosis takes place in the adrenal medulla but not in the

adrenal cortex. Therefore, this differential expression strongly suggests that adseverin relates not to the regulation of the secretory process in general but exclusively to the secretory process depending on the mode of exocytosis. Further, the localization of adseverin in the subplasmalemmal region agrees with the idea that this protein relates to the regulation of exocytosis.

Example 11: Construction of cDNA library originating in human kidney mRNA

As the human kidney mRNA, use was made of a product purchased from CLONTECH Laboratories, Inc. From 2 μg of this mRNA, double stranded cDNAs were synthesized by using TimeSaver™ cDNA Synthesis Kit (Pharmacia) in accordance with the attached protocol.

Namely, the thermally denatured mRNA was added to First-Strand Reaction Mix containing murine reverse transcriptase and oligo$(dT)_{12-18}$ primers and kept at 37°C for 1 hours to thereby synthesize the first strand. Next, the reaction mixture was added to Second-Strand Reaction Mix containing E. coli RNAaseH and E. coli DNA polymerase I and kept at 12°C for 30 minutes and then at 22°C for 1 hour to thereby synthesize the second strand. Then the double stranded cDNA thus synthesized was fractionated in size by using Spun Column included in the above-mentioned kit or agarose electrophoresis. Thus a cDNA of about 400 bp or more (in the former case) of about 2 to 3 kbp (in the latter case) was taken up exclusively.

After ligating an adaptor (EcoRI/NotI adaptor) to one end and eliminating the unreacted adaptor with the above-

mentioned Spun Column, the cDNA was integrated into a vector. Two vectors were prepared therefor, namely, ExCell vector (λ ExCell EcoRI/CIP) purchased form Pharmacia and Lambda ZAP^{R}II vector (PREDIGESTED LAMBDA ZAP^{R}II/EcoRI/CIAP CLONING KIT) purchased from STRATAGENE. As the host *E. coli*, NM522 strain was used in the former case while XL1-Blue strain was used in the latter case. Then the cDNA thus integrated into the vector was subjected to packaging with the use of GIGAPACK^R II PACKAGING EXTRACT (STRATAGENE) in accordance with the attached protocol. Namely, Freeze/Thaw extract, Sonic extract and the DNA were mixed and kept at 22°C for 2 hours to give a cDNA library.

Example 12: cDNA library screening by plaque hybridization (hybridization with the use of bovine adseverin cDNA as probe)

Screening was carried out by reference to the standard method described by Samborrk, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning, Cold Spring Harbor Lab. (1988). Namely, phage plaques grown on an LB agar plate were transcribed onto a Hybond-N filter (Amersham), denatured with an alkali and then immobilized by UV irradiation. Pre-hybridization was effected by keeping this filter in a hybridization solution at 40°C for 3 hours. Subsequently, hybridization was effected by keeping the filter together with a ^{32}P-labeled, thermally denatured probe (about 1 μCi/ml) at 40°C for 16 hours. As a probe, use was made of a fragment excised from bovine adseverin cDNA (pSK-adseverin) with the use of PstI and NdeI and corresponding to almost the full length of the cDNA. The hybridization was effected under less stringent conditions, i.e., by using a hybridization solution containing 25% of

formamide (4×SSC, 50 mM HEPES, pH 7.0, 10 x Denhardt's solution, 100 μg/ml thermally denatured salmon sperm) [Institute of Medical Science, University of Tokyo, Carcinostatic Research Section, "Shin Saibo Kogaku Jikken Purotokoru (New Protocols for Cell Technological Experiments)", Saibo Kogaku (Cell Technology), 1993]. After the completion of the hybridization, the filter was washed with a 2 x SSC solution containing 0.1% of SDS at room temperature for 15 minutes twice. Next, it was further washed with a 1 x SSC, 0.1% SDS solution with slowly elevating temperature from room temperature until the background radioactivity disappeared. Then the filter was dried followed by autoradiography.

The probe was labeled with ^{32}P by using a Random Primer DNA Labeling Kit Ver. 2 (Takara Shuzo Co., Ltd.). In accordance with the attached protocol, about 100 ng of thermally denatured DNA was labeled by keeping at 37°C for 30 minutes together with the random primer 50 μCi [α-^{32}P] dCTP and Klenow fragment.

First, 1.6×10^5 plaques of the cDNA library constructed from the human kidney mRNA obtained in Example 11 were screened with the use of the bovine adseverin cDNA as a probe. Thus a positive phage clone was obtained.

Example 13: Subcloning of positive phage clone into plasmid vector

By using primers (CAGCTATGACCATGATTACGCCAA; ACGACGGCCAGTGAATTGCGTAAT) synthesized from the base sequence of the λ ExCell vector, the insert of the clone obtained in Example 12 was amplified [Institute of Medical Science, University of Tokyo, Carcinostatic Research Section, "Shin

Saibo Kogaku Jikken Purotokoru (New Protocols for Cell Technological Experiments)", Saibo Kogaku (Cell Technology), 1993], and cleaved with EcoRI. Then it was subcloned into the pUC18 plasmid vector which had been cleaved with EcoRI and dephosphorylated. The clone thus obtained was named pADa-17.

Example 14: cDNA library screening by plaque hybridization (hybridization with the use of pADa-17 as probe)

By using a library newly constructed from the human kidney mRNA in accordance with the method of Example 11 and having cDNAs of 2 to 3 kbp exclusively concentrated therein, plaque hybridization was carried out with using the clone pADa-17 as a probe and increasing the strictness (50% formamide-containing hybridization solution: other composition being the same as the one of Example 12) under the conventional conditions. The vector employed for the construction of the cDNA library was Lambda ZAP^R II vector (PREDIGESTED LAMBDA ZAP^R II/EcoRI/CIAP CLONING KIT) purchased from STRATAGENE, while XLI-Blue strain was employed as the host *E. coli*. The probe was labeled with ^{32}P in the same manner as the one described in Example 12. Namely, a fragment excised from the clone pADa-17 was electrophoresed on an agarose gel and purified and about 100 ng thereof was labeled with 50 μCi of [α-^{32}P] dCTP. After the completion of the hybridization, the filter was washed with a 2×SSC solution containing 0.1% of SDS at room temperature for 15 minutes twice. Next, it was further washed with a 0.5×SSC, 0.1% SDS solution at 50°C for 15 minutes twice. Then the filter was dried followed by autoradiography.

Thus 5 positive phage clones were obtained by screening 1.7×10^5 plaques.

Example 15: Subcloning of positive phage clone into plasmid vector

From the positive phage clones, excision was carried out into a plasmid [pBluescript^R SK(-) vector] with the use of ExAssist^{TM}/SOLR^{TM} SYSTEM by taking advantage of the characteristics of the Lambada ZAP^R II vector. In accordance with the protocol attached to PREDIGESTED LAMBDA ZAP^R II/EcoRI/CIAP CLONING KIT (STRATAGENE), *E. coli* XL-1Blue strain was infected with the positive phages obtained in Example 14 and the ExAssist^{TM} helper phage and incubated at 37°C for 2.5 hours. Then the plasmid excised into the culture medium were incorporated into *E. coli* SOLR strain. Thus plasmid clones phAD-2 to 6 were obtained.

Example 16: Identification of base sequence of human adseverin cDNA

The base sequences of the plasmid clones phAD-2 and phAD-4 obtained in Example 15 were identified. The base sequences were identified by performing dideoxy sequencing with the use of Sequence Version 2.0 (United States Biochemical) or by the cycle sequencing with the use of PRISM^{TM} Terminator Mix (Applied Biosystems) and coding with the use of a Model 373A sequencer (Applied Biosystems).

The base sequence of human adseverin cDNA obtained by assembling the base sequences of phAD-2 and phAD-4 identified above and the amino acid sequence corresponding to the longest open reading frame are shown in SEQ ID NO: 5 in Sequence Listing.

Thus an open reading frame, which had the initiation codon at ATG at the 79-position and was composed of 715 amino acids, was found out.

Example 17: Comparison of human adseverin with bovine adseverin

Fig. 9 shows the result of a comparison between the amino acid sequence of human adseverin obtained in Example 16 and the amino acid sequence of bovine adseverin obtained in Example 6. In Fig. 9, the upper and lower columns correspond respectively to the human amino acid sequence and the bovine amino acid sequence. These amino acid sequences are completely identical with each other at the amino acids with the mark * and highly analogous at the amino acids with the mark ·. The human adseverin and the bovine adseverin show a homology of about 92% at the amino acid level and are highly analogous in many amino acids even though they are not completely the same. Although a high homology of about 90% is observed at the base level too, the homology shows a rapid decrease after the stop codon.

Example 18: Preparation of anti-adseverin antibody and anti-peptide antibody (antibody against human adseverin-derived peptide)

PREPARATION OF ANTI-ADSERVERIN ANTIBODY

1 mg of adseverin purified from bovine adrenal medulla was mixed with Freund's complete adjuvant to thereby give an emulsion. This emulsion was subcutaneously injected into a rabbit in ten and several parts. Moreover, the same amount of the protein was mixed with Freund's incomplete adjuvant and

the obtained emulsion was subcutaneously injected in the same manner at intervals of 4 weeks. 1 week after the injection, blood was collected from the ear vein and the serum was separated. When the antibody titer was determined by ELISA, an increase in the antibody titer of the serum was observed after the second or third booster. Since a cross reaction with gelsolin was observed, the serum was absorbed by gelsolin immobilized on agarose beads and then absorbed by immobilized adseverin. Next, it was eluted successively with 0.1 M glycine-HCl (pH 2.5), 0.1 M triethylamine-HCl (pH 11.5) and 3.5 M $MgCl_2$, dialyzed against Tris buffer salt solution and concentrated. The affinity purified antibody thus obtained showed no cross reaction with gelsolin but a reaction specific to adseverin. This antibody was used at concentrations of 0.1 to 1 μg/ml in the immunoblotting method and 1 to 10 μg/ml in the fluorescent antibody method.

PREPARATION OF ANTI-PEPTIDE ANTIBODY (ANTIBODY AGAINST HUMAN ADSEVERIN-DERIVED PEPTIDE)

Selection was made of 2 peptide sequences (16 residues) at sites which were exposed on the surface of protein molecules, had been very well conserved beyond difference in species between bovine adseverin and human adseverin and less homologous with gelsolin (SEQ ID NO: 6, 7). Staring from a resin having a branched structure to which 7 lysine residues were bound, a multiple antigen peptide (MAP) was synthesized (Tam, J.P., Proc. Natl. Acad. Sci. USA 85:5409-5413, 1988). Then emulsions were prepared by using this peptide with Freund's complete adjuvant in the first time and Freund's incomplete

adjuvant in the second time and thereafter. These emulsions were subcutaneously injected into 2 rabbits at intervals of 1 week. After 7, 8 and 9 weeks, blood was collected from the ear vein and the antibody titer was determined by ELISA. Thus an antibody, which showed scarcely any cross reaction with gelsolin and reacted with rat, bovine and human adseverins, was obtained. Since a nonspecific reaction shown in the unimmunized serum was observed, affinity purification was carried out similar to the case of the antibody obtained by immunizing with a purified protein.